

AWARD NUMBER: W81XWH-13-2-0097

TITLE: Proteomic Analysis of Trauma-Induced Heterotopic Ossification Formation

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Fort Detrick, Maryland 21702-5012

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[SF298]

Note: An abstract is required to be provided in Block 14

Over the past decade, improved personal protective equipment and medical support has reduced combat fatalities substantially among wounded war fighters. As a result, survivors are more likely to present with severe trauma to their arms and legs that will need multiple reconstructive surgeries or amputation during their recovery. The orthopaedic doctors caring for these wounded service personnel have been concerned by the fact that over 60% of these patients go on to form abnormal bone within the soft tissue of their injured limbs. This condition, known as Heterotopic Ossification (HO), causes pain, loss of mobility, and often requires additional surgeries to remove the rock hard tissue that has replaced their fat and muscle. While there are theories to explain why HO might occur, doctors still do not fully understand the mechanism(s) causing this disorder. Without knowing the mechanism, doctors find it difficult to predict which patients might be at risk for developing HO or to decide which drugs or treatments to use that would prevent HO from happening in these patients. The currently available treatments for HO have many undesirable side effects which can complicate the overall recovery process. The Specific Aims of this Idea Development proposal address these important questions by using blood samples collected from wounded warriors and civilians with bone injuries. The study will compare the blood samples between patients who either have or have not developed HO during the first year after their injury. The first experiments will ask, does the blood or wound fluid contain any proteins that can stimulate fat or muscle cells to form bone in the laboratory? This will test whether patients with HO have factors circulating in their blood or around the wound that specifically stimulate bone formation as compared to patients without HO. If this proves true, it will be an important step forward in understanding how HO occurs. The second experiments will ask, what is the identity of the protein(s) in the HO blood that might cause bone to form? The study will use a state of the art technique that can analyze all of the proteins in the blood and find out which ones are present. Using computer technology, researchers can then learn the name and function of these proteins of interest. This type of information will be of particular value to the orthopaedic surgeons caring for HO patients. The presence or absence of these proteins in the blood can be used to predict which patients might develop HO or to monitor HO treatment. Also, by knowing the names of the proteins involved in HO, doctors and pharmacists might be able to tell which drugs can be used to prevent HO formation at the time of injury. Wounded warriors and civilians would benefit directly from these advances since doctors would be able to prevent HO with a pill or drug or, at the very least, reduce the number of surgeries required to treat the condition when it happens. There would be minimal risk to wounded war fighters and civilian patients enrolled in this study. Patients would only be required to provide several extra tablespoons of blood to doctors during the weeks to months following their injury. This might cause a bruise but no other complications and would not interfere with their recovery in any way. It is predicted that this information could be used to improve patient care within 5 years or less after the study is completed. As a result, war fighters recovering from blast injuries in the future will have a better outlook than today's combat casualties. They will no longer have the same high risk of developing HO and can avoid the emotional, psychological

and physical damage sustained as a result of multiple orthopaedic surgical procedures. As a result, the effort, time, and cost of wounded warrior's recovery from life threatening orthopaedic trauma could be substantially reduced and as such, accelerate their return to active duty.

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- 1. INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Heterotopic Ossification (HO), the ectopic formation of bone in soft tissues, has been found to complicate >60% of extremity war injuries in casualties from Afghanistan and Iraq. Elevated levels of circulating and local cytokines released in response to high energy blast injuries have been found to correlate with the onset of HO, indicating that the disease process will require early intervention; however, preventive therapies such as tissue radiation, bone morphogenetic protein antagonists, and cyclooxygenase inhibitors, carry substantial risks for patients recovering from orthopaedic trauma. Consequently, there is an as yet unmet medical need to develop assays of serum and wound fluid biomarkers to identify those patients at greatest risk of HO progression during their recovery. The current project is using liquid chromatography/mass spectroscopy, in combination with other cell and protein biological assays, to evaluate the serum and wound fluid from civilian and military orthopaedic trauma patients for the presence of cytokines or factors capable of inducing HO. The studies are focusing on a select set of candidate biochemical pathways (bone morphogenetic, cyclic AMP, Wnt) that have been implicated in genetic models of HO. The research team includes expertise in civilian and military orthopaedic surgery, adipose and bone marrow stromal/stem cell biology, proteomics and mass spectroscopy, and regenerative medicine. During the first year of the project, the scope of the study has expanded to include the analysis of serum samples from established murine (burn) and rat (blast injury) models of HO and these analyses will be used to complement and support the initially proposed studies of human serum.

- 2. KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Adenylate Cycle (AC)
Adipose-derived Stromal/stem Cells (ASC)
Bone Marrow-derived Stromal/stem Cells (BMSC)
Bone Morphogenetic Protein (BMP)
Fibrodysplasia Ossificans Progressiva (FOP)
Heterotopic Ossification (HO)
Liquid Chromatography Mass Spectroscopy (LC/MS)
Progressive Osseous Heteroplasia (POH)
Skeletal Muscle Stromal/stem Cells (SMSC)
Wnt Pathway

OVERALL PROJECT SUMMARY: Summarize the progress during appropriate reporting period (single annual or comprehensive final). This section of the report shall be in direct alignment with respect to each task outlined in the approved SOW in a summary of Current Objectives, and a summary of Results, Progress and Accomplishments with Discussion. Key methodology used during the reporting period, including a description of any changes to originally proposed methods, shall be summarized. Data supporting research conclusions, in the form of figures and/or tables, shall be embedded in the text, appended, or referenced to appended

manuscripts. Actual or anticipated problems or delays and actions or plans to resolve them shall be included. Additionally, any changes in approach and reasons for these changes shall be reported. **Any change that is substantially different from the original approved SOW (e.g., new or modified tasks, objectives, experiments, etc.) requires review by the Grants Officer's Representative and final approval by USAMRAA Grants Officer through an award modification prior to initiating any changes.**

Training Specific Task 1: Mentoring of Dr. O'Brien

Current Objective: This Nested IDEA award included a component relating to the mentoring of Fred O'Brien MD. The intent is to advance Dr. O'Brien's training as a clinician scientist.

Results, Progress and Accomplishments with Discussion: During the past year, Dr. O'Brien has completed classwork during the Fall 2014 semester. In February, 2015, Dr. O'Brien visited the Tulane University program and presented grand rounds to the Department of Orthopaedic Surgery at LSUHSC-NO. Shortly thereafter, he was deployed to active duty in Afghanistan, returning to Ft. Gordon in late September, 2015. Dr. O'Brien has re-enrolled in classes in the Masters in Clinical Research Program at Tulane University for the Fall 2015 semester.

Research Tasks 2 & 3 (Military and Civilian Serum Samples Collection and Inventory):

Current Objective: The intent of these Tasks is to use an existing biorepository of serum samples from orthopaedic trauma patients with and without HO from the Walter Reed National Military Medical Center-Bethesda and to begin developing a comparable biorepository from a similar aged civilian population at the Louisiana State University Health Sciences Center-New Orleans.

Results, Progress and Accomplishments with Discussion: During the past year, the following tasks have been achieved or initiated:

1. IRB and HRPO approval of all aspects of the study at all of the involved sites has been renewed.
2. Samples from the inventory at Walter Reed National Military Medical Center-Bethesda have been evaluated at the Tulane University site. These human serum samples have been immunodepleted and shipped to the mass spectroscopy laboratory at Ohio State University for analysis.
3. Ms. Elaine Boos, study coordinator in the Dept. of Orthopaedic Surgery, LSUHSC-NO, has implemented protocols and procedures for civilian HO serum collection. To date, a total of nineteen subjects have been recruited and twelve remain actively enrolled. Six of these subjects have developed radiographic evidence of HO in less than one year following the initial trauma event.
4. An IRB protocol has been approved at LSUHSC-NO to perform a retrospective clinical study to assess frequency of heterotopic ossification in civilian orthopaedic trauma and elective surgical patients. Dr. Harry Molligan (PGY2, LSUHSC-NO) and Matt Fury (Medical Student LSUHSC-NO) are actively participating in this project and have begun to evaluate patient records.
5. Serum samples collected from murine (burn) and rat (blast injury) models of HO under approved IACUC protocols have been incorporated into the study design.

The inclusion of these animal models will provide opportunities to better control the degree of injury and the timing of serum collection and, more important, will provide the team with access to serum samples that can be replaced (unlike the more limited and therefore valuable human specimens). Dr. Benjamin Levi (University of Michigan) has provided access to his murine burn model of HO while the rat blast injury model has been developed in the laboratories of Drs. Davis and Forsberg at Naval Medical Research Center.

Research Tasks 4-6 (Osteoconductive and Osteoinductive Biochemical and Cell Based Assay Evaluation with Serum Stimulation):

Current Objective: The intent of these Tasks is to develop biochemical and cell based assays for detection of factors activating signal transduction pathways associated with osteogenesis.

Results, Progress and Accomplishments with Discussion: During the past year, the following tasks have been achieved or initiated:

1. Dr. Martin in collaboration with Ms. Claire Llamas (Tulane) and Dr. Ammar Qureshi (NMRC) is nearing completion of PCR and western blot studies with human ASC following induction with BMP, forskolin, PMA and IL6 exposure as well as pooled human serum from military and civilian subjects with or without HO development. Studies have identified the AP1 transcription factors and the MAPK/ERK signal transduction pathway as early responsive biomarkers of osteogenic differentiation in the ASC. Parallel studies are underway using the same inductive factors to evaluate the response of human bone marrow mesenchymal stem cells (BMSC) and human muscle derived stromal cells. Additionally, Dr. Martin and Dr. Qureshi are completing studies evaluating the expression of microRNA regulatory proteins in human ASC and human BSMC, respectively.
2. In addition to the ASC isolated from healthy tissue donors, Dr. Martin will evaluate cells isolated from burn patients at risk for HO. An IRB protocol to Tulane University School of Medicine to work with de-identified ASC from patients treated by Dr. Levi and his colleagues under an approved IRB at the University of Michigan remains pending but has had HRPO approval. Dr. Martin and Ms. Llamas will culture these samples at the Center for Stem Cell Research and Regenerative Medicine and evaluate their expression of osteogenic and inflammatory biomarkers based by qRT-PCR and immunoblot assays.

Research Task 7 (Manuscript and Oral Presentation of Biochemical and Cell Studies):

Current Objective: The intent of this Task is to disseminate information gained through the biochemical and cell based assays relating to osteogenesis to the general scientific community.

Results, Progress and Accomplishments with Discussion:

Dr. Martin, along with Dr. Gimble (Tulane) has submitted a review article on miRNA in the context of adipogenesis, myogenesis, and osteogenesis with Dr. Ammar Qureshi (post-doctoral fellow) and Dr. Tom Davis at NMRC, Dr. Vin Dasa at LSUHSC-NO, and Dr. Mike Freitas at OSU. The manuscript has just been

published electronically in a special issue of Biochimie focusing on aging, metabolism and obesity.

Martin EC¹, Qureshi AT², Dasa V³, Freitas MA⁴, Gimble JM¹, Davis TA⁵ MicroRNA regulation of stem cell differentiation and diseases of the bone and adipose tissue: Perspectives on miRNA biogenesis and cellular transcriptome. Biochimie. 2015 Feb 26. pii: S0300-9084(15)00047-4.

A manuscript that reports that AP1 transcription factors and the MAPK/ERK signal transduction pathway may be early responsive biomarkers for early bone development is nearing completion and will be submitted for peer review to the Journal of Bone and Joint Surgery (Am).

A manuscript reporting the human BMSC and skeletal muscle HO and signaling studies will be prepared and submitted for peer review, likely to the Journal of Bone and Joint Surgery (Am).

Two manuscripts reporting the expression of micro RNA processing apparatus in human ASC and human BMSC will be prepared for submission to the journal Adipocyte and Bone, respectively.

Research Task 8 (Preparation and Proteomic Analysis of Serum Samples):

Current Objective: The intent of this Task is to develop LC/MS assays for detection of factors activating signal transduction pathways associated with osteogenesis.

Results, Progress and Accomplishments with Discussion: During the past year, the following tasks have been achieved or initiated:

1. Human serum samples obtained from military (NMRC) and civilian (LSUHSC-NO) subjects with and without HO have been immunoselected using a commercial kit by Ms. Claire Llamas (Tulane University). These samples have been shipped to Dr. Michael Freitas and his graduate student (Michael Hoover) for mass spectroscopy.
6. A panel of rat serum samples from blast injured and amputated animals have been prepared by Dr. Qureshi and Dr. Davis at NMRC, shipped to Tulane, immunoselected and then transferred to OSU for mass spectroscopy. Preliminary mass spectroscopy data from the rat HO model has identified and quantified time dependent changes in the profiles of 280 individual proteins. These specimens are currently being further evaluated using mass spectroscopy approaches with greater sensitivity. Bioinformatic approaches to correlate changes in protein levels to the time course of HO development are underway. Similar approaches will be carried out with pooled human HO serum samples from early, intermediate, and late collection time points as well as the controlled time points from the murine burn HO model.
2. Serum samples obtained by Dr. Levi (University of Michigan) from mice subjected to burns over 30% of their body surface area and Achilles tendonotomy to initiate HO in 100% of the treated animals have been immunoselected (by Ms. Llamas, Tulane) and shipped to Ohio State University. These samples, along with their appropriate controls, will be evaluated by LC/MS

using new instrumentation following completion of the human and rat sample analyses.

Research Task 9 (Manuscript and Oral Presentation of Proteomic Studies):
Nothing to report.

3. KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research. Project milestones, such as simply completing proposed experiments, are not acceptable as key research accomplishments. Key research accomplishments are those that have contributed to the major goals and objectives and that have potential impact on the research field.

a. Mentoring – Return of Dr. Fred O'Brien from deployment and enrollment in the Master's Program.

b. Research –

- i. Publication of review article on the role of micro RNA in ASC and BMSC differentiation in a special issue of the journal *Biochimie*.
- ii. Department of Orthopaedic Surgery Grand Rounds presentation at LSUHSC-NO by Dr. O'Brien (New Orleans LA, February, 2015)
- iii. Poster presentations by Dr. Martin at the Extremity Wound Injury Symposium (Washington DC, January, 2015) and the Military Health System Research Symposium (Ft. Lauderdale FL, August 2015).

4. CONCLUSION: Summarize the importance and/or implications with respect to medical and /or military significance of the completed research including distinctive contributions, innovations, or changes in practice or behavior that has come about as a result of the project. A brief description of future plans to accomplish the goals and objectives shall also be included.

The second year of this research project has continued to integrate productive and complementary activities across four distinct campuses. Critical assays necessary to complete the study goals are well established, validated, and effectively shared between campuses. The team is advancing its experimental data to peer reviewed publication. The outcomes of the experiments now underway and/or nearing completion have begun to define biomarkers and signal transduction pathways involved in the onset of HO. These are being investigated and validated across species (human, murine, rat) and will advance our understanding of the signal transduction pathways regulating osteogenesis in bone and soft tissue derived stromal/stem cells. In the coming year, the investigative team will seek to achieve the following goals:

1. Publication of primary research manuscripts regarding the response of human ASC, BMSC and skeletal muscle cells to osteogenic inductive factors and

human serum from HO and non-HO civilian and military patients in a time dependent manner.

2. Complete a detailed LC/MS study of serum biomarkers in the rat and human with accompanying manuscript submissions. Similar studies on the murine HO serum proteome will be initiated shortly thereafter.
3. Initiate miRNA analyses in serum samples of at least one specie in the context of HO formation.
4. Continue to present abstracts for poster and oral presentation at regional, national and international meetings from the investigative team. Dr. Martin will be scheduled to present her work at the upcoming International Federation of Adipose Therapeutics and Science (IFATS) meeting in New Orleans (November 2015). She and others will continue to consider other presentation venues.

5. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

- a. List all manuscripts submitted for publication during the period covered by this report resulting from this project. Include those in the categories of lay press, peer-reviewed scientific journals, invited articles, and abstracts. Each entry shall include the author(s), article title, journal name, book title, editors(s), publisher, volume number, page number(s), date, DOI, PMID, and/or ISBN.

(1) Lay Press:

None

(2) Invited Articles:

None

(3) Peer Reviewed Articles:

Martin EC, Qureshi AT, Dasa V, Freitas MA, Gimble JM, Davis TA. MicroRNA regulation of stem cell differentiation and diseases of the bone and adipose tissue: Perspectives on miRNA biogenesis and cellular transcriptome. Biochimie. 2015 Feb 26. pii: S0300-9084(15)00047-4.

(4) Abstracts:

EC Martin, A Qureshi, AG King, PC Krause, OC Lee, V Dasa, JA Forsberg, TA Davis, EA Elster, MA Freitas, JM Gimble
Regulators of Bone Formation induce early response genes in human mesenchymal adult stem/stromal cells.

EC Martin, AT Qureshi, AG King, PC Krause, OC Lee, V Dasa, MA Freitas, JA Forsberg, EA Elster, TA Davis, JM Gimble
Pro-Inflammatory and Osteo-inductive Cytokines Induce Bone-Related Early Response Genes in Adult Human Mesenchymal Stem Cells

- b. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.
- i) January 2015: Extremity Wound Injury X (Sponsored by AAOS/OTA/SOMOS/ORS). Poster by Elizabeth Martin PhD.
Title: Regulators of Bone Formation induce early response genes in human mesenchymal adult stem/stromal cells.
Authors: EC Martin, A Qureshi, AG King, PC Krause, OC Lee, V Dasa , JA Forsberg, TA Davis, EA Elster, MA Freitas, JM Gimble
- ii) August 2015: Military Health System Research Symposium. Poster by Elizabeth Martin PhD.
Pro-Inflammatory and Osteo-inductive Cytokines Induce Bone-Related Early Response Genes in Adult Human Mesenchymal Stem Cells
EC Martin, AT Qureshi, AG King, PC Krause, OC Lee, V Dasa, MA Freitas, JA Forsberg, EA Elster, TA Davis , JM Gimble

6. INVENTIONS, PATENTS AND LICENSES: List all inventions made and patents and licenses applied for and/or issued. Each entry shall include the inventor(s), invention title, patent application number, filing date, patent number if issued, patent issued date, national, or international.

Nothing to report.

7. REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. This list may include development of prototypes, computer programs and/or software (such as databases and animal models, etc.) or similar products that may be commercialized.

Nothing to report.

8. OTHER ACHIEVEMENTS: This list may include degrees obtained that are supported by this award, development of cell lines, tissue or serum repositories, funding applied for based on work supported by this award, and employment or research opportunities applied for and/or received based on experience/training supported by this award.

Completion of Overseas Deployment by Dr. Fred O'Brien.

For each section, 4 through 9, if there is no reportable outcome, state "Nothing to report."

9. REFERENCES: No references cited.

APPENDICES: No appendices attached.

NOTE:

TRAINING OR FELLOWSHIP AWARDS: For training or fellowship awards, in addition to the elements outlined above, include a brief description of opportunities for training and professional development. Training activities may include, for example, courses or one-on-one work with a mentor. Professional development activities may include workshops, conferences, seminars, and study groups.

Dr. O'Brien has completed one semester of classwork in the Masters of Clinical Science Research Program at Tulane University School of Medicine and has re-enrolled online since returning from his deployment.

COLLABORATIVE AWARDS: Not applicable.

QUAD CHARTS: Quad Chart submitted with annual report

MARKING OF PROPRIETARY INFORMATION: Not applicable.

Proteomic Analysis of Trauma-Induced Heterotopic Ossification Formation

ERMS/Log Number OR120163 Task Title

Award Number W81XWH-13-2-0097



PI: Jeffrey Gimble MD PhD

Org: Tulane University Award Amount: \$962,052

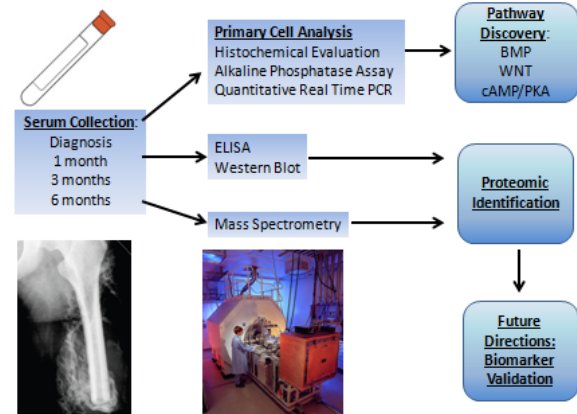
Study/Product Aim(s)

- Mentor Dr. O'Brien as Clinical Research Scientist
- Recruit civilian & military orthopaedic trauma study population
- Harvest serial serum & wound fluid samples from subjects at risk for HO development
- Examine clinical fluids for osteogenic activity using in vitro assays
- Identify potential HO biomarkers using proteomic assays

Approach

Orthopaedic trauma patients from civilian and military hospitals will provide serial serum and wound fluid samples. These will be shared between collaborating laboratories in assays examining (a) activation of osteogenic biochemical pathways in human primary adipose, bone marrow and skeletal muscle cells and (b) proteome identification using mass spectroscopy, ELISA, and western immunoblotting technologies.

Heterotopic Ossification Biomarker Discovery



Accomplishment: (1) Rat HO blast injury serum mass spectroscopy analyses identifying 280 proteins; (2) Biochemical and cell based assays for osteogenic signal transduction pathways; (3) Review article in *Biochimie*; (4) Abstracts at two national meetings

Timeline and Cost

Activities	CY	13	14	15	16
Mentoring – Fred O'Brien MD					
IRB/CRADA/MTA - HRPO					
Recruit/Inventory Study Subjects					
Assay Development & Analyses					
Estimated Budget (\$K)		\$87.5	\$339.5	\$307	\$228

Goals/Milestones

CY13 Goal – HRPO Evaluation, Assay Development, Mentoring

- ☒ HRPO documents submitted, Assay development & enrollment in Masters in Clinical Research Program initiated

CY14 Goals – In Vitro Assay Development, Mentoring

- ☐ Complete clinical fellowship & complete initial Master's classes
- ☐ Develop primary cell culture assays & mass spec controls
- ☐ Review article submissions

CY15 Goal – Civilian Patient Accrual & Assay Data Collection

- ☐ Meet enrollment goals for civilian patient accrual
- ☐ Identify target biomarkers in serum & wound fluid

CY16 Goal – Manuscript Submission, Mentoring, Final Data Analyses

- ☐ Complete primary research article submissions to peer review journals
- ☐ Completion of Master's Program
- ☐ Final analyses of data to determine HO biomarkers in serum

Comments/Challenges/Issues/Concerns

- HRPO completion determines when patient enrollment begins.

Budget Expenditure to Date

Projected Expenditure: \$600K

Actual Expenditure: Approximately 75%; 4th Year No Cost Extension

Anticipated

Updated: 17-Jun-2014